【DOCUMENT NAME】 Specification

【TITLE OF INVENTION 】 NICOTIANAMINE SYNTHASE AND GENE ENCODING THE SAME

【CLAIMS】

【CLAIM 1】 A nicotianamine synthase comprising amino acid sequence shown in SEQ ID NO: 1, or amino acid sequence having deletion in a part thereof, being substituted by the other amino acids or being added with the other amino acids.

【CLAIM 2】 The nicotianamine synthase according to claim 1 wherein said enzyme is originated from barley.

【CLAIM 3】 The nicotianamine synthase according to claim 1 or 2 comprising having amino acid sequence shown in SEQ ID NO: 1, 3, 5, 7, 9, 11 or 13.

【CLAIM 4】 The nicotianamine synthase according to claim 1 wherein said enzyme is originated from Arabidopsis.

【CLAIM 5】 The nicotianamine synthase according to claim 1 or 4 comprising having amino acid sequence shown in SEQ ID NO:15, 17 or 19.

【CLAIM 6】 A gene encoding amino acid sequence of nicotianamine synthase according to any one of claims 1 - 5.

【CLAIM 7】 The gene according to claim 6 wherein said gene is cDNA.

【CLAIM 8】 The gene according to claim 6 or 7 comprising having base sequence shown in SEQ ID NO: 2, 4, 6, 8, 10, 12 or 14.

【CLAIM 9】 The gene according to claim 6 or 7 comprising having base sequence shown in SEQ ID NO: 16, 18 or 20.

【CLAIM 10】 A vector comprising containing gene

according to any one of claims 6 - 9.

【CLAIM 11】 The vector according to claim 10 wherein said vector is an expression vector.

【CLAIM 12】 A transformant wherein said transformant is transformed by the vector according to claim 10 or 11.

【CLAIM 13】 The transformant according to claim 12 wherein the foreign gene is a gene having base sequence shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, or 20.

【CLAIM 14】 The transformant according to claim 12 or 13 wherein the host is bacteria.

【CLAIM 15】 The transformant according to claim 12 or 13 wherein the host is higher bacteria.

【CLAIM 16】 A process for production of nicotianamine comprising using the transformant according to any one of claims 12 - 15.

【CLAIM 17】 A plant wherein the gene according to any one of claims 6 - 9 is introduced.

【CLAIM 18】 The plant according to claim 17 wherein said plant is seed.

【CLAIM 19】 A fruit obtained by growing the plant according to claim 17 or 18.

【CLAIM 20】 An antibody against nicotianamine synthase according to any one of claims 1 - 5.

【CLAIM 21】 The antibody according to claim 20 wherein said antibody is polyclonal antibody.

【CLAIM 22】 The antibody according to claim 20 wherein said antibody is monoclonal antibody.

【CLAIM 23】 A method for extraction of nicotianamine synthase comprising extracting the said enzyme in the presence

of thiol protease inhibitor at the extraction of nicotianamine synthase from the plant.

【CLAIM 24】 The method according to claim 23 wherein the thiol protease inhibitor is E-64.

【Detailed Of Invention】

【0001】

【FIELD of Invention】

【0002】

The present invention relates to a nicotianamine synthase involved in the mugineic acid biosynthetic pathway, the amino acid sequence thereof, a gene encoding the same, a vector, a process for producing nicotianamine by using the same, plants transformed by the gene encoding the nicotianamine synthase, and an antibody against the nicotianamine synthase.

【PRIOR ART】

【0003】

Graminaceous plants that absorb by chelating the insoluble state Fe(III) in soil using mugineic acid and adopt so called the Strategy-II mechanism of Fe acquisition secrete Fe chelators (phytosiderophores) from their roots to solubilize sparingly soluble Fe in the rhizosphere (Roemheld, 1987). The amount of the secreted phytosiderophores increases under Fe-deficiency stress. The mugineic acid family is the only examples of phytosiderophores known so far (Takagi, 1976). Tolerance to Fe deficiency in graminaceous plants is thought to depend on a quantity of mugineic acid family secreted by plants (Takagi et al. 1984, Roemheld and Marschner 1986, Marschner et al. 1987, Mori et al. 1987, Kawai et al. 1988, Mori et al. 1988, Mihashi and Mori 1989, and Shingh et al. 1993).

【0004】

The biosynthetic pathway of mugineic acid in plants is shown in Fig. 1. S-adenosylmethionine is synthesized from methionine by S-adenosylmethionine synthase. Subsequently, three molecules of S-adenosylmethionine are combined to form one molecule of nicotianamine by nicotianamine synthase. The generated nicotianamine is then converted to 3" -keto acid by nicotianamine aminotransferase, and 2' -deoxymugineic acid is synthesized by the subsequent action of a reductase. A further series of hydroxylation steps produces the other mugineic acid derivatives including mugineic acid from the deoxymugineic acid (Mori and Nishizawa 1987, Shojima et al. 1989, Shojima et al. 1990 and Ma and Nomoto 1993).

A compound in Fig. 1, a compound in the lower right, wherein R1 and R2 are hydrogen and R3 is hydroxyl, is mugineic acid. A compound wherein R1 is hydrogen and R2 and R3 are hydroxyl, is 3-hydroxymugineic acid. Also a compound wherein R2 is hydrogen and R1 and R3 are hydroxyl, is 3-epihydroxymugineic acid.

【0005】

Three S-adenosylmethionine synthase genes were isolated from barley roots, but these genes were not induced by Fe deficiency (Takizawa et al. 1996). A gene Ids3, which is obtained from the barley by differential screening, is suspected to be a gene, which converts deoxymugineic acid to mugineic acid by hydroxylation and is strongly induced by Fe-deficiency (Nakanishi et al. 1993). Further, nicotianamine aminotransferase was purified and isolated from Fe-deficient barley roots, and two nicotianamine aminotransferase genes, Naat-A and Naat-B, were isolated (Takahashi et al. 1997). Naat-A expression was induced in Fe-deficient roots.

【0006】

The synthesis of nicotianamine from S-adenosylmethionine is similar to polyamine synthesis from decaroboxy-S-adenosylmethionine. In contrast to polyamine synthase, however, nicotianamine synthase catalyzes the combination of three S-adenosylmethionine molecules and the azetidine ring formation at the same time (Fig. 1). Such the nicotianamine synthase is a novel type of enzyme. Previously, we reported the partial purification of nicotianamine synthase from the roots of Fe-deficient barley and expression pattern of the activity (Higuchi et al. 1994, Higuchi et al. 1995, Kanazawa et al. 1995, Higuchi et al. 1996a and Higuchi et al. 1996b). Since nicotianamine synthase is easily decomposed during extraction and purification, it has been difficult to purify sufficient quantities for amino acid sequencing.

【0007】

【Problem to be solved by the invention】

The present invention has an object to provide a plant, especially graminaceous plant, highly tolerant to Fe-deficiency, as a result of isolating and purifying a nicotianamine synthase, being cloned the gene of this enzyme, determining the base sequence and amino acid sequence thereof, and using said enzyme.

【0008】

【Means for solving problem】

The present invention relates to a nicotianamine synthase shown in SEQ ID NO: 1 comprising amino acid sequence shown in SEQ ID NO: 1, or amino acid sequence having deletion in a part thereof, being substituted by the other amino acids or being added with the other amino acids.

The present invention relates to the gene encoding said amino acid sequence of nicotianamine synthase.

The present invention also relates to a vector comprising containing said gene, and a transformant transformed by the said vector.

【0009】

The present invention relates to a process for production of nicotianamine using the said transformant.

The present invention further relates to plants, especially graminaceous plants, to which said gene is introduced, and fruits obtained by growing said plants.

The present invention relates to a process for extraction of said nicotianamine synthase in the presence of thiol protease inhibitor, preferably E-64.

Further, the present invention relates to an antibody against said nicotianamine synthase.

【0010】

We have tried to isolate nicotianamine synthase (Higuchi et al. Plant & Soil, Vol. 165, p. 173-179, 1994), and since nicotianamine synthase was easily decomposed and was difficult to isolate and purify, we were unable to obtain sufficient amounts of protein to determine its partial amino acid sequence. Subsequently, it was found that a thiol protease inhibitor E-64 (hereinafter designates as E-64) was very effective in suppressing degradation of nicotianamine synthase (Higuchi et al. Plant & Soil, Vol. 178, p. 171 - 177, 1996 a).

In the present invention, as a result that frozen roots were crushed to a fine powder in liquid N2 and then rapidly homogenized with buffer containing 0.1 mM thiol protease inhibitor E-64, nicotianamine synthase protein could be

isolated and its gene could also be isolated.

【0011】

Further, the enzyme of the present invention recovered its activity by removal of SDS after SDS-PAGE treatment, but the rate of recovery was very low (Higuchi et al. Plant & Soil, Vol. 165, p. 173-179, 1994). Consequently, degree of purification should be increased up before treatment of SDS-PAGE. Then the column chromatography procedures were further improved.

We have also found that the enzyme of the present invention is relatively hydrophobic and a buffer containing a mild surface active agent CHAPS increased the rate of recovery. Several ion-exchange chromatography carriers were tested, and DEAE-Sepharose FF and DEAE Sephacel were found to be the most effective. In addition to TSK gel Butyl Toyopearl, another hydrophobic chromatography carrier, TSK gel Ether Toyopearl 650M, effectively removed impurities of the 30 - 35 kDa.

【0012】

The enzyme of the present invention has been reported that it was the peptide of 30 - 35 kDa, the activity of which was recovered by removing SDS after SDS-PAGE treatment, and the activity was detected as a broad molecular weight range of 30 - 35 kDa (refer to Fig. 3). Fig. 3 shows a result of preparative SDS-PAGE in the fractions showing enzyme activity. SDS-PAGE was carried out using 11% acrylamide slab gels. A portion of the gel was stained with Coomassie brilliant blue and the rest of the gel was stained with Cu. The gel containing proteins between 30 - 35 kDa in size was cut into seven fragments (indicated by the short lines). The thick bars in Fig. 3 indicate relative enzymatic activities detected from each gel fragment.

In order to identify nicotianamine synthase peptide from

the proteins having these molecular weights, the peptides, which were contained in the nicotianamine synthase fractions, purified from Fe-deficient and control barley roots were compared using SDS-PAGE. From each barley root 200 g, the present enzyme was purified according to the method described in example 3 hereinbelow.

【0013】

The enzyme activity of the control was a quarter of the Fe-deficient roots.

The peptide composition of the active enzyme fraction from each purification step of the present enzyme was analyzed and compared by SDS-PAGE, and results are shown in Fig. 2. Fig. 2 show comparison with the active fraction from the purification step of Fe-deficient barley roots 200 g [in the figure, shown with (-)], and the active fraction from the purification step of the control barley roots 200 g [in the figure, shown with (+)]. SDS-PAGE was carried out using 12.5% acrylamide slab gels (Laemmli, Nature Vol. 227, p. 680-685, 1970). Gels were stained with Coomassie brilliant blue. Fig. 2[A] shows a step before DEAE-Sepharose. The upper row shows enzyme from Fe-deficient barley roots and the lower row shows enzyme from control roots. In each lane, lanes 1, crude extract, 200μg of protein; lanes 2, after Butyl Toyopearl 650M, 100μg of protein; lanes 3, after hydroxyapatite, 20μg of protein; and lanes 4, after Butyl Toyopearl 650M, 15μg of protein, are shown.

Fig. 2[B] shows after DEAE-Sepharose FF, each lane, 25 μg of protein. Fig. 2[C] shows after Ether Toyopearl 650M; in which left shows inactive fraction, and right shows active fraction, and 1/25 of each fraction is electrophoresed.

【0014】

As a result, almost no difference was observed in both Fe-deficient and control roots before DEAE-Sepharose step (refer to Fig. 2[A]). After the DEAE-Sepharose step it became clear that the 30- and 31-kDa peptides were induced by Fe-deficiency (refer to Fig. 2[B]). After the Ether Toyopearl step, the 31 kDa peptide was eliminated from the active fraction. The 32 and 33 kDa peptides were found to be newly induced by Fe-deficiency (refer to Fig. 2[C]). Activities were detected from the 32 and 33 kDa peptides, but no activity was detected from 30 kDa peptide (refer to Fig. 3).

【0015】

Molecular weight of the enzyme of the present invention was determined by gel-filtration.

Estimated molecular weight of nicotianamine synthase by gel-filtration was reported to be 40,000 - 50,000 (Higuchi et al. Plant & Soil, Vol. 165, p. 173-179, 1994). But this did not correspond with the value estimated by SDS-PAGE.

In the present study, the buffer containing CHAPS effectively increased the resolution and molecular weight of the present enzyme was estimated to be 35,000 (refer to Fig. 4). This corresponds well to the value estimated by SDS-PAGE.

【0016】

Fig. 4 shows elution pattern of nicotianamine synthase from the gel-filtra

tion column. The black circles (●) indicate the enzyme activity and the solid line indicates absorption at 280 nm. The active fraction after hydroxyapatite chromatography was applied to a Sephacryl S300HR (Pharmacia) column (1.5 cm × 71 cm, 125 ml), equilibrated with developing buffer (50 mM Tris, 1 mM EDTA, 0.1 M KCl, 0.05% CHAPS, 0.1 mM p-APMSF and 3 mM DTT,

pH 8.0). Molecular weight markers used were thyroglobulin (Mr 670,000), γ-globulin (Mr 158,000), ovalbumin (Mr 44,000), and myoglobin (Mr 17,000). The linear flow was 10 cm/hour.

【0017】

Partial amino acid sequence was determined from purified nicotianamine synthase.

The above explained 30 kDa, 32 kDa and 33 kDa peptides were purified from 1 kg of Fe-deficient barley roots by using a method in example 3 hereinbelow. These were partially degraded using a method in example 4 hereinbelow. Although 32- and 33-kDa peptides could not be completely separated from each other, these might have similar sequence or 32 kDa peptide was presumed to be the degradation product of 33 kDa peptide, and both of them were degraded in together.

The determined partial amino acid sequences indicated that these peptides were very similar in each other (Fig. 5). Further, since the molecular weights of the 33 kDa and 32 kDa (1) fragments had almost unchanged molecular weight as compared with before degradation, this sequence might be N-terminal region of the present enzyme. As a result of computer search of the database, a gene of unknown function having very similar sequence to these sequences was found to exist in Oryza sativa and Alabidopsis thaliana. Especially, EST-cDNA clones D23792 and D24790 of Oryza sativa were very similar with 80.0% identity in a 33-amino acid overlap in the former and 68.4% identity in a 19-amino acid overlap in the latter (Fig. 5).

Fig. 5 shows a comparison with a six partial amino acid sequence determined by nicotianamine synthase originated from barley and similar sequence of graminaceous plants obtained by computer search of the database. Identical amino acid residue

is shown in ":" . The part of nucleotide sequences indicated by the arrows was applied for the sequences of primer used in PCR.

【0018】

Cloning and nucleotide sequences of cDNA clones encoding nicotianamine synthase were performed and determined.

PCR amplification of total cDNA prepared from Fe-deficient barley roots using degenerate primers designed from the partial amino acid sequence obtained from the method explained hereinbefore was performed, but the objective DNA could not amplified. Then the primers having single nucleotide sequence (shown by arrows in Fig. 5) from sequences of Oryza sativa, D23792 and D24790, were synthesized and PCR amplification was performed. The 205 bp fragment was amplified by PCR using NF and NR primers and the 274 bp fragment was amplified by PCR using IF and IR primers, and these contained the objective sequences. A cDNA library prepared using poly (A) + RNA from Fe-deficient barley roots was screened and 19 positive clones using the 205 bp fragment probe and 88 positive clones using the 274 fragment bp probe were obtained.

【0019】

Among the thus obtained clones, the clone designated as NAS1, contained a translated region of 985 bp and amino acid sequence deduced therefrom was 328 amino acids residue, with deduced molecular weight of 35,144. This corresponded well with the value estimated by SDS-PAGE and gel-filtration. The partial amino acid sequences of the 32 kDa and 33 kDa peptides were included totally in NAS1 (Fig. 6).

Fig. 6 shows full length of NAS1 cDNA and amino acid sequence deduced therefrom. The underlined sequences indicate

the identical partial amino acid sequences of fragments in the above Fig. 5. Numbers of the nucleotide sequence are indicated to the right of each row. Amino acid numbers are indicated on the left of each row.

The predicted pI of 5.2 matched the value estimated by native isoelectric focusing electrophoresis well. The six clones having very similar sequence other than NAS1, i.e. NAS2, NAS3, NAS4, NAS5-1, NAS5-2 and NAS6, were also obtained (Table 1, Fig. 7).

Fig. 7 shows comparison of the deduced amino acid sequences of the above 7 cDNA obtained from barley. Asterisks "*" indicates identical amino acid residues in all sequences.

The nucleotide sequences of these clones are shown in SEQ ID NO: 2 (NAS1), SEQ ID NO: 4 (NAS2), SEQ ID NO: 6 (NAS3), SEQ ID NO: 8 (NAS4), SEQ ID NO: 10 (NAS5-1), SEQ ID NO: 12 (NAS5-2) and SEQ ID NO: 14 (NAS6), respectively. The amino acid sequences of these amino acid sequences are shown in SEQ ID NO: 1 (NAS1), SEQ ID NO: 3 (NAS2), SEQ ID NO: 5 (NAS3), SEQ ID NO: 7 (NAS4), SEQ ID NO: 9 (NAS5-1), SEQ ID NO: 11 (NA5-2) and SEQ ID NO: 13 (NAS6), respectively.

【0020】

【Table 1】

【0021】

The partial amino acid sequences determined from the 30 kDa peptide were all included in NAS5. The 5' - and 3' -non-translated regions of these clones were not similar with each other.

However, except 122-140 amino acid residues of NAS5-2, NAS5-1 and NAS5-2 were identical. NAS5-1 is thought to be the one derived from the NAS5-2, part of which was deleted. However,

it has not been made clear at present whether this phenomenon occurred in the plant body or in the process of the experiment.

D23792 and D24790 similar to nicotianamine synthase of Oryzae sativa were found with about 80% identity to HvNAS1. AC003114 and AB005245 of Arbidopsis thaliana were found with about 45% identity to HvNAS1.

Three types of nicotianamine synthase genes of Arbidopsis thaliana were obtained by using primer(shown in under line part in figure) for genome DNA of Arbidopsis thaliana and operating PCR.

The nucleotide sequences are shown in SEQ ID NO: 16 (AtNAS-1), SEQ ID NO: 18 (AtNAS-2)and SEQ ID NO: 20 (AtNAS-3), respectively. The amino acid sequences of these amino acid sequences are shown in SEQ ID NO: 15 (AtNAS-1), SEQ ID NO: 17(AtNAS-2)and SEQ ID NO: 19 (AtNAS-3), respectively.

【0022】

The obtained NAS1 protein was expressed in E. coli.

The PCR amplification of NAS1 ORF was cloned with vector pMAL-c2 to express NAS1 fused with C-terminal of maltose biding protein. The expression of fused protein is strongly induced by IPTG.

The crude extract was obtained from the transformed E. coli, and nicotianamine synthase activity was assayed in the state of the fused protein. The crude extract from the strain transformed with only the vector could not be detected the activity, whereas in case of inserted with NAS1 ORF, the activity was detected. Result is shown in Fig. 8.

Fig. 8 shows results of thin layer chromatographic (TLC) analysis of nicotianamine synthase obtained from E. coli crude extract expressing a fused protein of maltose binding protein

- NAS1. In Fig. 8, lane 1: a standard nicotianamine synthase; lane 2: E. coli expressing maltose binding protein (SAM); and lane 3: E. coli expressing maltose binding protein - NAS1 fused protein.

【0023】

Northern hybridization analysis conducted by the method described in example 7 hereinbelow indicated that this gene was strongly induced in Fe-deficient roots (Fig. 9). This coincides with expression pattern of the present enzyme activity (Higuchi et al. 1994). Fig. 9 shows a result of Northern hybridization analysis using HvNAS1 as a probe. Total RNA was extracted from after one week of Fe-deficient treatment and control barley leaves and roots, and in each lane, 5 μg of RNA were electrophoresed.

【0024】

Southern hybridization analysis of the barley genome DNA was performed according to the method described in example 8 hereinafter mentioned. Cutting of DNA with BamHI, EcoRI or HindIII produced plurality of fragments, however none of clones obtained at present could be digested by BamHI and EcoRI, consequently nicotianamine synthase gene might exist with multiple copies in genomes of barley and rice (Fig. 10).

Fig. 10 shows Southern - hybridization analysis of NAS1 as a probe. Genomic DNAs from barley and rice were digested with BamHI (lanes B), EcoRI (lanes R) and HindIII (lanes H) and 10 μg thereof were electrophoresed in each lane.

【0025】

Further, using antigen prepared by the method described in example 9 hereinbelow, Western-blot analysis was performed according to the method described in example 10. It was found

that the present enzyme protein was rapidly decomposed during the operation in the crude extract prepared for detecting the present enzyme activity (Fig. 11). The staining patterns coincided with the fact that the present enzyme activity was detected on the broad range between 30 - 35 kDa after SDS-PAGE (refer to Fig. 3).

Fig. 11 shows Western-blot analysis of crude enzyme used for detection of activity. SDS-PAGE was performed using 12.5% acrylamide slab gel. Protein 100μg was electrophoresed.

【0026】

The crude extract obtained from denatured protein according to the method described in example 10 hereinbelow was detected as almost single band with 35 - 36 kDa (Fig. 12). This value coincided with the deduced value from the amino acid sequence.

Fig. 12 shows Western-blot analysis of total protein extracted by trichloroacetic acid/acetone. SDS-PAGE was performed using 12.5% acrylamide slab gel. Protein 100μg was electrophoresed. Proteins 200μg extracted from roots and proteins 500μg extracted from leaves were electrophoresed.

Western-blot analysis after 2-dimention electrophoresis reveals to detect several spots. This coincided with the fact of obtaining plurality of nicotianamine synthase gene. All spots were induced in Fe-deficient roots.

【0027】

The amount of secreted mugineic acid is reported increased up to 20 mg mugineic acid/g roots dry weight/day (Takagi, 1993). Crude nicotianamine synthase activity detected by the present inventors was sufficient to fulfill it. Since the present enzyme proteins exist in more than several types and 30 kDa peptide

without activity exists, it can be speculated that as a result of aggregation of these peptides, the constructed structure, which is preferable for binding with 3 molecules of S-adenosylmethionine, reveals maximum activity. The molecular weight estimated by gel-filtration was 35,000 (Fig. 4).

Increase in activity by re-aggregation of subunits has not been observed at present. Since the fused protein with maltose binding protein and subunits showed its activity, we have at present an idea that the present enzyme might be a monomer. However, the possibility that large activity can be revealed by constructing multimer, can not be completely denied.

【0028】

The reaction mechanism synthesizing nicotianamine from S-adenosylmethionine may be similar to methyl transfer reaction using S-adenosylmethionine as a methyl donor, and a reaction synthesizing spermidine and spermine from decarboxylated S-adenosylmethionine. The common catalytic domain of these enzymes has been discussed in relation to equivalent amino acids configuration occupying similar positions in higher-order structures (Hashimoto et al. 1998 and Schluckebier et al. 1995).

In future, catalytic domain may be elucidated as the results of comparison with nicotianamine synthase from other plant species or X-ray crystallography.

【0029】

Induction of nicotianamine synthase activity by Fe-deficiency is a specific phenomenon in graminaceous plants, and is essential for mass production of mugineic acid family. Oryza sativa is a plant, in which secretion of mugineic acid family is the least among major graminaceous plants, consequently it is very weak for Fe-deficiency in calcareous

soil.

Consequently, as a result of creating transformant Oryza sativa having tolerance to Fe-deficiency by introducing nicotianamine synthase gene of the present invention into the graminaceous plants, especially Oryza sativa, and expressing large amount at the Fe-deficiency, cultivation of rice in the calcareous soil can be possible.

Heretofore, in the graminaceous plants, nicotianamine has been thought to have only a role as a precursor for synthesis of mugineic acid family. However, since the present invention has elucidated that nicotianamine synthase gene constituted the multiple gene family, it may play other important roles in the graminaceous plants.

【0030】

In plants, which lack the ability to secrete mugineic acid family, except for graminaceous plants, it has been proposed that nicotianamine plays a key role as an endogenous chelator of divalent metal cations, such as Fe2+, Cu2+, Zn2+ and Mn2+, and that it contributes to the homeostasis of those metals (Stephan et al. 1994). Consequently, it may play the same role in the graminaceous plants.

Nicotianamine synthase activity is not induced in dicots, and expression of gene of the present invention may not be induced by Fe-deficiency. We have cloned nicotianamine synthase genes of Arabidopsis thaliana. Composition of promoter regions in these genes can elucidate the mechanism of gene expression caused by Fe-deficiency, and the gene of the present invention may play important function not only in the graminaceous plants but also in the dicots.

【0031】

SEQ ID NO: 1 shows amino acid sequence of nicotianamine synthase of the present invention.

The present invention includes nicotianamine synthase having amino acid sequence shown in SEQ ID NO: 1. However, the present invention is not limited within the above nicotianamine synthase. The nicotianamine synthase of the present invention includes, unless it loses nicotianamine synthase activity, the peptides, in which a part of the amino acid sequence of said peptide is deleted, preferably 50% or less, more preferably 30% or less, or more further preferably 10% or less in the total amino acids, or is substituted by other amino acids, or to which other amino acids are further added, or in which these deletion, substitution and addition may be combined.

【0032】

Nucleotide sequence coding nicotianamine synthase of the present invention is shown in SEQ ID NO: 2.

The present invention also includes not only a gene coding nicotianamine synthase shown in SEQ ID NO: 2 but also genes coding nicotianamine synthase mentioned hereinabove.

【0033】

The vector of the present invention introducing the above gene is not specifically limited, and various vectors can be introduced. Preferable vector is the expression vector.

Various cells can be transformed conventionally by using recombinant vector of the present invention. Mass production of nicotianamide can be performed by using the thus obtained transformant. These methods are well known in the person skilled in the art.

【0034】

Examples of hosts for introducing the gene of the present

invention are bacteria, yeasts and cells. Preferable host is plants, especially the graminaceous plant.

Method for introducing gene is not limited. It can be made by using vector or can be directly introduced in genome.

【0035】

Antibody of the present invention against nicotianamine synthase can be prepared conventionally by using nicotianamine synthase of the present invention. Antibody can be a polyclonal antibody or, if necessary, monoclonal antibody.

【0036】

Further, a selective breeding of plants, preferably graminaceous plants, can be made by using gene of the present invention. Especially, the gene of the present invention can be applied for improvement of varieties, which can grow even in Fe-deficient soil.

【0037】

【Examples】

The following examples illustrate the present invention, but are not construed as limiting the present invention.

【0038】

Example 1. (Preparation of plant material)

Seeds of barley (*Hordeum vulgare* L. cv Ehimehadakamugi No. 1) were germinated on wet filter paper and transferred into the standard hydroponic culture solution (Mori and Nishizawa, 1987) in a glass house at natural temperature under natural light. The pH of the hydroponic culture solution was adjusted at 5.5 by 0.5 N HCl everyday. When the third leaves developed, the plants were transferred to the hydroponic culture solution without containing Fe. The pH was maintained at 7.0 by 0.5 N NaOH everyday. The control plants were also cultured in the

standard culture solution continuously. The culture solution was renewed once in every week. Two weeks after starting Fe-deficient treatment, when severe iron chlorosis significantly appeared on the 4th and 5th leaves, roots were harvested and frozen in liquid N2 and stored at −80℃ until use.

【0039】

Example 2. (Assay of nicotianamine synthase activity)

Modified assay method reported previously by the present inventors (Higuchi et al. 1996a) was used. Enzyme solutions were equilibrated with reaction buffer [50 mM Tris, 1 mM EDTA, 3 mM dithiothreitol (hereinafter designates as DTT), 10 μ M (p-amidinophenyl) methanesulfonyl fluoride (hereinafter designates as p-APMSF) and 10 μ M trans-epoxysuccinyl-leucylamido-(4-guanidino) butane (hereinafter designates as E-64), pH 8.7]. Buffer exchange was performed by using ultrafiltration unit, Ultrafree C3LGC NMWL10000 (Millipore Co.). S-adenosylmethionine labeled with 14C in carboxyl group (Amersham Inc.) was added to the enzyme solution at the final concentration of 20μM and kept at 25℃ for 15 minutes. The reaction products were separated by thin layer chromatography on silica gel LK6 (Whatman Inc.) using developer (phenol : butanol : formic acid : water = 12 : 3 : 2 : 3). Radioactivity of the reaction products was detected by image Analyzer BAS-2000 (Fuji Film Co.). The protein content was assayed by Bradford method using Protein Assay Kit (Bio Rad Inc.).

【0040】

Example 3 (Purification of nicotianamine synthase)

The following operations were performed at 4℃ and E-64 was added to fractions containing nicotianamine synthase at the

final concentration of 10μM.

The frozen roots were crushed into a fine powder in liquid N2 and homogenized in a household juicer with 200 ml of extraction buffer [0.2 M Tris, 10 mM EDTA, 5% (v/v) glycerol, 10 mM DTT, 0.1 mM E-64, 0.1 mM p-APMSF and 5% (w/v) insoluble polyvinylpyrrolidone (PVP), pH 8.0] per 100 g of roots. The homogenate was centrifuged for 30 minutes at 22,500 × g to obtain supernatant. Ammonium sulfate was added to the supernatant to yield a final concentration of 0.4 M and allowed to stand for 1 hour. Again, the mixture was centrifuged for 30 minutes at 22,500 × g to obtain supernatant.

【0041】

The supernatant was loaded onto a TSK gel Butyl Toyopearl 650M column (10 ml bed volume per 100 g of roots), equilibrated with the adsorption buffer [20 mM Tris, 1 mM EDTA, 3 mM DTT, 0.4 M (NH4)2SO4 and 0.1 mM p-APMSF, pH 8.0] and eluted with elution buffer [10 mM Tris, 1mM EDTA, 3 mM DTT, 0.1 mM p-APMSF, 5% glycerol and 0.05% 3-[(3-chloramidopropyl) dimethyl-ammonio] propanesulfonic acid (hereinafter designates as CHAPS), pH 8.0].

【0042】

KCl was added to the active fraction to give a final concentration of 0.4 M, and 1 M potassium phosphate buffer (pH 8.0) was added to a final concentration of 1 mM of KCl. A hydroxyapatite 100 - 350 mesh (Nacalai Tesque), equilibrated with the adsorption buffer (1 mM K-P, 10 mM KCl, 3 mM DTT and 0.1 mM p-APMSF, pH 8.0), was prepared at 10 ml per protein 100 mg and the fractions containing nicotianamine synthase were loaded. Nicotianamine synthase was passed through without adsorption. The passed through fraction was loaded onto TSK gel

Butyl Toyopearl 650M column (1 ml bed volume per 10 mg of protein), and nicotianamine synthase was eluted in the manner described above.

The active fraction was loaded onto a DEAE-Sepharose FF column (5 ml bed volume per 25 mg of protein, Pharmacia) equilibrated with the adsorption buffer (20 mM Tris, 1 mM EDTA, 3 mM DTT, 0.1 mM p-APMSF and 0.05% CHAPS, pH 8.0) and eluted with stepwise gradient elution of potassium chloride concentration of 0.05 M, 0.1 M, 0.15 M and 0.2 M. Nicotianamine synthase was eluted at 0.15 M of KCl concentration.

【0043】

The active fraction was loaded onto the Ether Toyopearl 650M column (10 ml bed volume per 100g of roots), equilibrated with adsorption buffer [20 mM Tris, 1 mM EDTA, 3 mM DTT, 1.2 M (NH4)2SO4 and 0.1 mM p-APMSF, pH 8.0]. Nicotianamine synthase was not adsorbed and passed through from the column. The passed through fraction was loaded onto TSK gel Butyl Toyopearl 650M column and fractions containing nicotianamine synthase was eluted. The peptides in the active fraction containing nicotianamine synthase, which was purified by the above column chromatographic treatments, were separated by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (hereinafter designates as SDS-PAGE) using 11% acrylamide slab gels. After SDS-PAGE the gel was stained with 0.3 M copper chloride (Dzandu et al. 1988), and the separated bands were cut out. The gel fragments were destained with 0.25 M EDTA/0.25 M Tris (pH 9.0) and homogenized with the extraction buffer (1% SDS, 25 mM Tris and 192 mM glycine). Each homogenate was electroeluted with SDS-free buffer (25 mM Tris and 192 mM glycine) and peptide was recovered.

【0044】

Example 4. (Determination of partial amino acid sequence)

The isolated nicotianamine synthase was digested chemically with cyanogen bromide (Gross 1967).

After SDS-PAGE treatment, 10-fold volume of 70% (v/v) formic acid and 1% (w/v) cyanogen bromide were added to gel fragments containing nicotianamine synthase and decomposed at 4℃ for overnight. After completion of digestion, the liquid part was collected and dried in vacuo. The dried substance was dissolved in SDS-PAGE sample buffer, and allowed to stand at room temperature for overnight, then the digested product was separated by SDS-PAGE using 16.5% acrylamide gel containing Tricine (Schagger and Jagow, 1987). The peptides were transferred onto a PVDF membrane by electroblotting (Towbin et al. 1979) and stained with amido black. The stained bands were cut out and the amino acid sequence was determined from N-terminal side of each peptide by Edman degradation in gas-phase sequencer (model 492A protein sequencer, Applied Biosystems Inc.).

【0045】

Example 5. (Cloning of nicotianamine synthase genes)

PCR amplification was conducted for cDNA originated from Fe-deficient barley roots using primers, which were synthesized based on the obtained partial amino acid sequence. A pYH23 cDNA library prepared from the poly (A)+RNA of Fe-deficient barley roots was screened with the thus obtained DNA fragments of PCR product, which was labeled with [α-32P]dATP using the random primer kit (Takara Shuzo Co.), as the primers. The isolated cDNA clones were sequenced by cycle sequencing kit (Shimadzu Bunko Co.) using Shimadzu DNA sequencer DSQ-1000L.

PCR amplification was conducted for genomic DNA of Arabidopsis thaliana using primers, which were synthesized based on nucleotide sequences of AC003114 and AB005245 of Arabidopsis thaliana. The thus obtained DNA fragments were sequenced by cycle sequencing kit (Shimadzu Bunko Co.) using Shimadzu DNA sequencer DSQ-1000L.

The determined nucleotide sequence is shown in SEQ ID NO: 2.

【0046】

Example 6. (Expression of NAS1 protein in E. coli)

A fragment, in which EcoRI site was introduced into the upstream of the first ATG of the HvNAS1 cDNA and PstI and BamHI sites were introduced into the downstream of the stop codon of the HvNAS1 cDNA, was amplified by PCR. The thus obtained amplified product was subcloned in the pBluescriptII SK- using EcoRI site and BamHI site, and the correct nucleotide sequence was confirmed. The fragment between EcoRI site and PstI site was cloned into pMAL-c2 to make expression in the form of fusing the HvNAS1 to the C-terminal of maltose binding protein.

【0047】

E. coli strain XL1-Blue was used as a host for expressing the said fused protein. pMAL-c2-NAS1 and pMAL-c2, respectively, were introduced into XL1-Blue. The thus obtained recombinant bacteria were cultured in LB medium containing ampicillin and tetracycline, each 50 μg/ml, at 37℃ until the OD 600 of the culture reached 0.5. Isopropyl β -D-thiogalactopyranoside (IPTG) was added to the final concentration of 0.3 mM, and continuously cultured at 37℃ for 3 hours, and collected bacterial cells. Cells were suspended in 10 mM Tris buffer containing 0.2 M NaCl, 1 mM EDTA, 3 mM DTT and 0.1 mM E-64, pH

7.4 and frozen with liquid nitrogen. This was melted in ice water and ultrasonication for 15 seconds was repeated for 10 times. Nicotianamine synthase activity of the thus obtained crude extract was assayed according to the method described in example 2 and the enzyme activity was confirmed.

【0048】

Example 7. (Northern hybridization)

Northern hybridization of barley RNA was performed using DNA fragment, which was prepared by cutting NAS1 cDNA with HindIII and NotI and labeled with [α-32P]dATP, as a probe. Total RNA was extracted from barley (Naito et al. 1988). The extracted RNA was separated by 1.4% agarose gel electrophoresis, and blotted onto Hybond-N+ membranes (Amersham). The membrane was hybridized with the probe in 0.5 M Church phosphate buffer (Church and Gilbert 1984), 1 mM EDTA, 7% (w/v) SDS with 100 μg/ml salmon sperm DNA at 65℃ for overnight. The membrane was washed with buffer containing 40 mM Church phosphate buffer and 1% (w/v) SDS at 65℃ for 10 minutes. After the washing was repeated once again, the membrane was washed with buffer containing 0.2 × SSPE and 0.1% (w/v) SDS at 65℃ for 10 minutes. Radioactivity was detected using the image analyzer BAS-2000.

Results are shown in Fig. 9.

【0049】

Example 8. (Southern hybridization)

Genomic DNA was extracted from leaves of barley and rice. The extract was digested with BamHI, EcoRI or HindIII, separated on a 0.8% (w/v) agarose gel electrophoresis, and transferred onto Hybond-N+ membranes (Amersham). The hybridization was performed according to the method described in example 7 and radioactivity was detected.

Result is shown in Fig. 10.

【0050】

Example 9 (Preparation of polyclonal antibody)

About 100 μg of isolated nicotianamine synthase was administered to two rats on antigen.

Sample by which partial amino acid sequence becomes clear was used as an antigen. The complete Freund adjuvant was used at the immunity of the first time and the incomplete Freund's adjuvant was used since the second times. After immunity had been done to the rat four times, rat's whole blood was collected blood. The obtained serum was preserved at -80℃.

【0051】

Example 10 (Western blotting analysis)

Total protein was extracted using trichloroacetic acid and acetone (Damerval et al. 1986). The plants were crashed in the liquid nitrogen until powder was obtained, and mixed with acetone containing 0.1% (v/v) 2-mercaptoethanol. The protein was precipitated by allowing to stand at -20℃ for 1 hour, and the precipitate was collected by centrifugation at 16,000 × g for 30 minutes. The precipitate was suspended in acetone containing 0.1% (v/v) 2-mercaptoethanol and allowed to stand at -20℃ for 1 hour. The precipitate was dried in vacuo, and dissolved in the sample buffer [9.5 M urea, 2% (w/v) Triton X-100 and 5% (v/v) 2-ME], then centrifuged at 16,000 × g for 10 minutes to obtain the supernatant. The proteins contained in the supernatant were separated by SDS-PAGE or the denaturing two-dimensional electrophoresis (O ' Farrell 1975) and transferred onto PVDF membrane. Western blotting analysis was performed by applying the primary antibody containing anti-nicotianamine synthase antibody prepared in example 1 and